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FUNGAL AND PARASITIC INFECTIONS

Acquired Clinical Immunity to Malaria in Nonhuman Primates Coinfected with *Schistosoma* and *Plasmodium* Parasites

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ABSTRACT Naturally acquired immunity to malaria develops over several years and can be compromised by concomitant infections. This study explored the influence of chronic schistosomiasis on clinical outcome and immunity to repeated malaria infection. Two groups of baboons (n = 8 each), were infected with *Schistosoma mansoni* cercariae to establish chronic infections. One of the two groups was treated with praziquantel (PZQ) to eliminate schistosome infection. The two groups plus a new malaria control group (n = 8) were inoculated three times with *Plasmodium knowlesi* parasites at 1-month intervals. Clinical data and IgG, IgG1, memory T-cell, and monocyte levels were recorded. After three *P. knowlesi* infections, we observed (i) reduced clinical symptoms in all groups with each subsequent infection, (ii) increased IgG and IgG1 levels in the malaria control (Pk-only) group, (iii) increased IgG, IgG1, $CD14^+$, and $CD14^-$ $CD16^+$ levels in the *Schistosoma*-treated (Schisto/PZQ+Pk) group, and (iv) significantly lower IgG and IgG1 levels compared to those of the Pk-only group, reduced $CD4^+$ $CD45RO^+$ levels, and increased levels of $CD14^-$ $CD16^+$ cells in the coinfected (Schisto+Pk) group. Chronic *S. mansoni* infection does not compromise establishment of clinical immunity after multiple malaria infections, with nonclassical monocytes seeming to play a role. Failure to develop robust antibody and memory T cells may have a long-term impact on acquired immunity to malaria infection.

KEYWORDS schistosomiasis, malaria, coinfection, acquired immunity

Malaria continues to be a significant contributor to high morbidity and mortality rates in the tropical and subtropical regions of the world, as do pathogenic helminth infections such as schistosomiasis. In 2018, the WHO reported an estimated 288 million cases of malaria infections worldwide, with approximately 405,000 deaths (1). With schistosomiasis, around 290.8 million people globally required preventative treatment, out of which 97.2 million were treated (https://www.who.int/news-room/fact-sheets/detail/schistosomiasis). Since the pathogens share the same geographical locations, coinfections of malaria and schistosomiasis are common and have been shown to have an impact on morbidity and transmission of both diseases (2, 3).

Exposure to *Plasmodium* infection over years often result in partial naturally acquired immunity mainly influenced by age and exposure patterns to infection. Studies have indicated that both the innate and adaptive host immune responses play a crucial role in mediating tolerogenic and antiparasitic protective mechanism resulting in the reduction of clinical symptoms and subsequent reinfection. Clinical immunity is manifested as reduced parasitemia and inflammatory reactions (4, 5). During the symptomatic stage of malaria infection, both human and animal studies have shown that $CD4^+$ T and B cells confer their effector functions by producing antibodies and promoting differentiation

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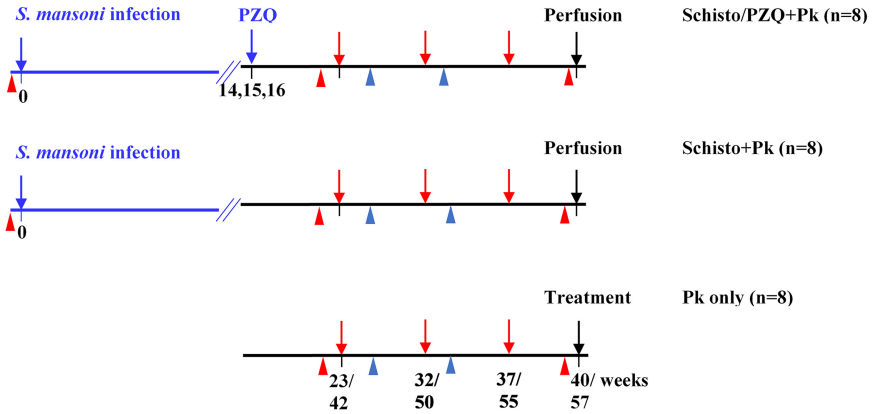

FIG 1 *S. mansoni* and *P. knowlesi* infection and treatment protocol. Blue arrows indicate *S. mansoni* infection and red arrows *P. knowlesi* infection. Praziquantel (PZQ) for schistosomiasis treatment was administered at weeks 14, 15, and 16 postinfection. For phase I, the first, second, and third *P. knowlesi* infections were at weeks 23, 32, and 37, respectively, while phase II infections were at weeks 42, 50, and 55. The end point for phase 1 was at week 40 and that for phase II at 57 weeks post schistosome infection. After each malaria infection, animals were treated with antimalarial drug (blue triangle). Blood for serum was collected (red triangles) before schistosome infection (week 0), before malaria infection (weeks 20 and 40 for phases I and II, respectively) and after three malaria infections (weeks 40 and 57). PBMC isolation was done before malaria infection and after three malaria infections.

into memory cells to facilitate parasite clearance (6–8). IgG antibodies, primarily IgG1 and IgG3 subtypes, are associated with acquired immunity to malaria infection and act by neutralizing or activating complement lysis of merozoites, antibody-dependent cell cytotoxicity by natural killer cell and monocyte activation (7–10). Upon activation, monocytes help in parasite clearance by phagocytosis, cytokine production, and antigen presentation. They are, however, also implicated in the production of a predominant inflammatory T helper 1 (Th1) immunity which, if not regulated, can lead to systemic inflammation and vascular dysfunction (11). Downregulation of the induced Th1 milieu is facilitated by production of interleukin 10 (IL-10), IL-27 and transforming growth factor beta (TGF-β), which lessen malaria-induced clinical symptoms and pathology (8, 12).

Chronic *S. mansoni* infection is associated with a potent immunoregulatory CD4$^+$ Th2 polarized immune response that limit immunopathology and help the parasite survive within the host (13). During coinfection, the Th2-polarized milieu can potentially impair the development of humoral immunity to *Plasmodium* infection resulting in higher parasitemia and greater morbidity to malaria infection (14, 15). Conversely, other studies have indicated that schistosome coinfection can provide protection against severe malaria (16–18). How such immune alterations affect naturally acquired immunity to malaria has not been extensively studied (19–21). We therefore performed a randomized controlled study to evaluate the impact of *S. mansoni* coinfection on acquired immunity to *P. knowlesi*. This study was done in baboons, which are natural hosts for *S. mansoni* (22) and a well-established model for severe infection with *P. knowlesi* (23).

RESULTS

Study population and animal survival. At the beginning of the study, 24 animals were randomized to three groups, namely, Schisto+Pk, Schisto/PZQ+Pk, and a malaria control group (Pk-only) (Fig. 1). Infection and treatment procedures are as described in Materials and Methods. At the beginning of phase II (weeks 32 and 40 post schistosome infection), two animals were excluded from the study, namely Pan 3733 (Schisto+Pk), due to death from intestinal intussusception, and Pan 3699 (Schisto/PZQ+Pk), due to injuries. The remaining 22 animals were infected with *P. knowlesi* parasites and monitored daily for malaria-related morbidity. Five animals succumbed to

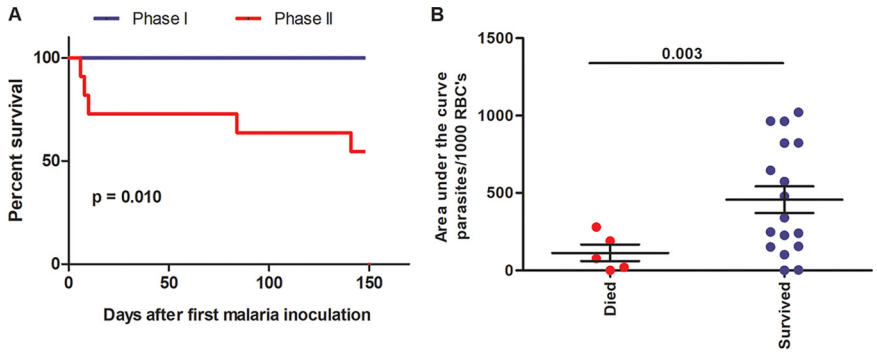

FIG 2 Increased mortality in phase II experiment. Five animals succumbed to malaria infection during the experimental period. (A) Percent parasitemia of these animals that died compared to those that survived is. (B) Survival outcome of animals during the two experimental phases. Differences in parasite density were analyzed using the Welch two-sample *t* test. Animal survival was analyzed using the log-rank test.

malaria disease at phase II of the study. Two animals from the Schisto+Pk group died 8 days after the first and 21 days after the second *P. knowlesi* infection, two animals from the Schisto/PZQ+Pk group died at 3 and 9 days after first infection, and one animal from the Pk-only group died at 17 days following the third infection (Fig. 2A). Although animals that died had significantly lower overall parasitemia (area under the curve [AUC]) at time of death compared to those that survived ($P = 0.0031$), they manifested severe clinical symptoms (Fig. 2B). Gross pathology from three animals in the groups exposed to schistosome infection had at least six of the following conditions: splenomegaly, hepatomegaly, pulmonary edema, ecchymosis along the groove of the heart, whipworms in the cecum, granulomas on the liver and large intestines, and/or congested kidney and brain vessels. Cause of death was attributed to metabolic disturbances, septicemia, and pneumonia.

Reduced malaria related morbidity in baboons after multiple infections with *P. knowlesi* parasites. We investigated clinical outcomes following each of three rounds of *P. knowlesi* infections (Fig. 3). After the first infection, all three groups of animals showed clinical signs of malaria, with loss of appetite being the most common. These signs diminished with subsequent rounds of *P. knowlesi* infection, such that after the third infection, 0 out of 5 infected (100%) in the Schisto+Pk, 2 of 5 (40%) in the Schisto/PZQ+Pk, and 3 of 8 (37.5%) in the Pk-only groups had signs of clinical malaria. Out of all the symptoms we observed, loss of appetite and fever decreased most dramatically after multiple infections (Fig. 3A and B). These results indicate that repeated exposure to *P. knowlesi* infection was associated with decreased clinical malaria symptoms, which were not affected by an ongoing or previous schistosome infection.

Multiple *P. knowlesi* infections is associated with decreased parasitemia. To determine infection intensity from *P. knowlesi* infection, parasitemia was measured as described in Materials and Methods and presented as AUC. After the first infection, 4 of 7 (57.1%), 4 of 7 (51.7%), and 7 of 8 (87.5%) in the Schisto+Pk, Schisto/PZQ+Pk, and Pk-only groups, respectively, failed to spontaneously clear *P. knowlesi* infection. After the second challenge, more animals were able to spontaneously clear or control their malaria infection by day 12, while 1 of 5 (20%), 3 of 5 (60%), and 3 of 8 (37.5%) in the Schisto+Pk, Schisto/PZQ+Pk, and Pk-only groups, respectively, had to be treated. By the third challenge, only 2 of 5 animals in the Schisto+Pk group, 3 of 5 in the Schisto/PZQ+Pk group, and 7 of 8 in Pk-only group developed parasitemia, which spontaneously cleared. There were no differences in parasitemia between the three groups at all time points. In the Pk-only group, there was a decrease in parasite density at the third infection (median = 562.8 parasitized red blood cells [iRBCs]) compared to the second (median = 76 iRBCs; $P = 0.044$) and first (median = 185.3 iRBCs; $P = 0.067$), as shown in Fig. 4L. Although not significant, there was reduction in parasitemia at the second (median = 4.6 iRBCs; $P = 0.160$) and third (median = 0.09 iRBCs; $P = 0.061$) infections

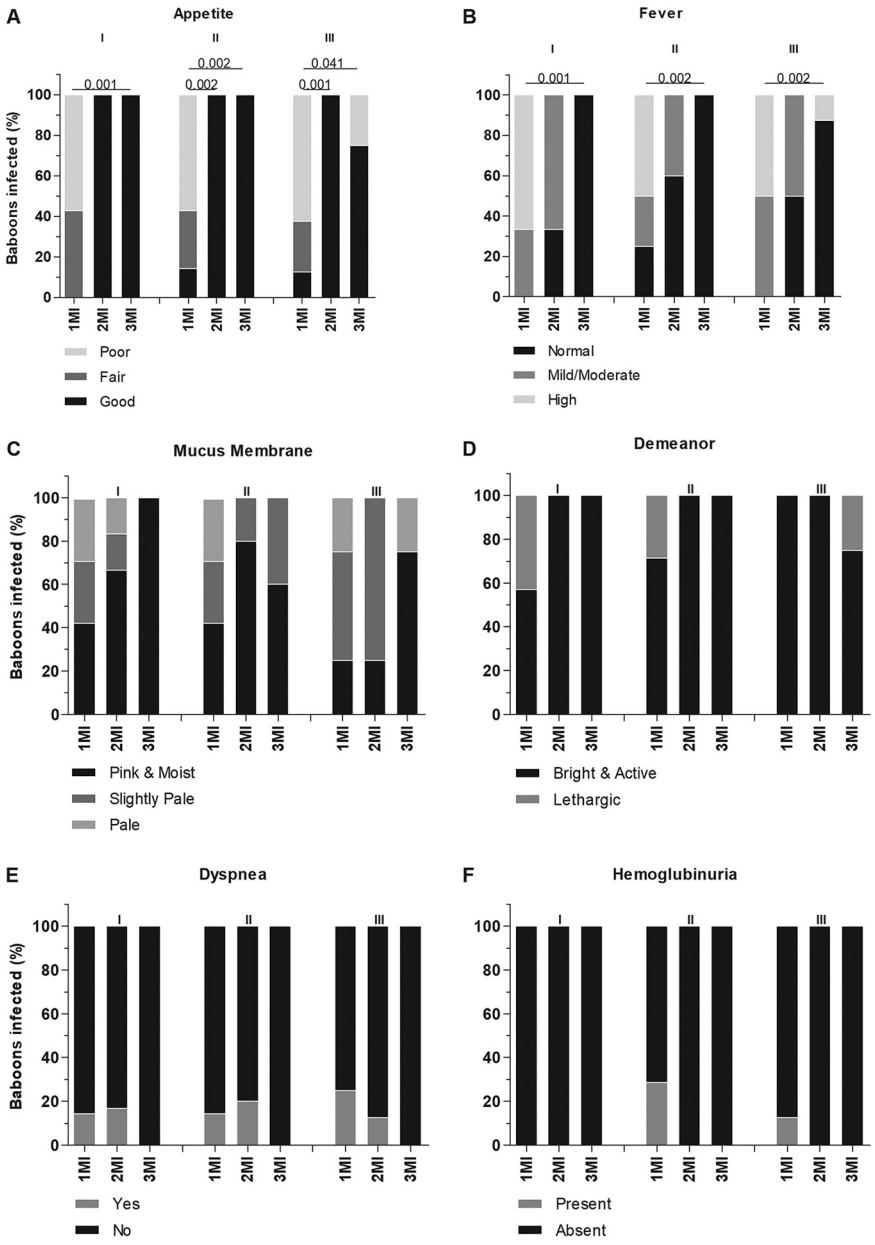

FIG 3 Reduced morbidity after three *P. knowlesi* infections. Animals were infected three times with malaria parasites, indicated as first (1MI), second (2MI), and third (3MI) infections. The bar graphs (A to F) represent proportions of animals with clinical symptoms of severe malaria in the Schisto+Pk (I), Schisto/PZQ+Pk (II), and Pk-only (III) groups. Statistical analysis within groups to identify differences in clinical severity after three malaria infections was done using chi-square analysis.

compared to the first (median = 248.5 iRBCs) in the Schisto+Pk group (Fig. 4D). Similarly, there was a decline in parasite levels in the Schisto/PZQ+Pk group at the third (median = 51.6 iRBCs) compared to first (median = 155 iRBC) and second (median = 382 iRBC) infections, although this was not significant (Fig. 4H). Based on these observations, protection from overwhelming parasitemia was achieved after multiple exposure to *P. knowlesi* infection, despite a previous or ongoing schistosome infection.

***S. mansoni* coinfection results in reduced specific IgG antibodies after multiple *P. knowlesi* infections.** IgG and IgG1 produced against crude *P. knowlesi* antigen were measured in serum of infected animals. IgG levels in the Schisto+Pk and Schisto/PZQ+Pk groups did not significantly change across all time points. This was in contrast with the Pk-only group, where IgG levels were significantly higher at third infection

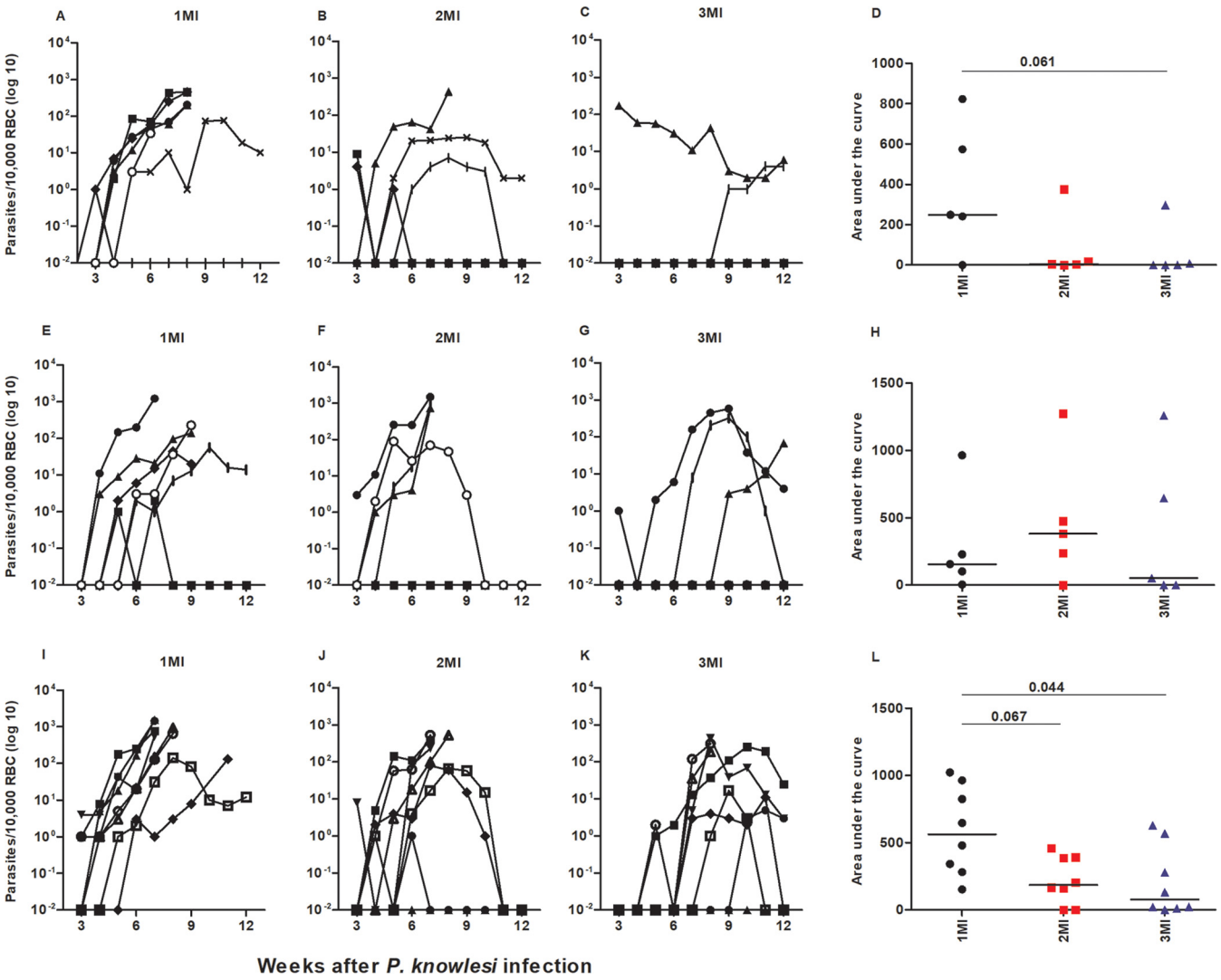

FIG 4 Reduced parasitemia after three *P. knowlesi* infections. Individual % parasitemia is presented as parasites/10,000 RBCs ($\log_{10}$). Daily parasitemia was recorded from days 3 to 12 after iRBC/10,000 malaria infection, shown on the *x* axis. The graphs represent parasitemia in the Schisto+Pk (A, B, and C), Schisto/PZQ+Pk (E, F, and G), and Pk (I, J, and K) at first (1MI), second (2MI), and third (3MI) infections. Area under the curve (AUC) during 1MI, 2MI, and 3MI up to the time of treatment is shown for Schisto+PK (D), Schisto/PZQ+Pk (H), and Pk-only (L), with the median shown. Differences in parasite density were measured as AUC was analyzed using Kruskall-Wallis with Dunn's *post hoc* analysis and multiple comparisons adjusted by the Holm method.

compared to the baseline (P = 0.001) and after first infection (P = 0.02), as shown in Fig. 5A. IgG1 levels, on the other hand, significantly increased at the third infection compared to the first in the Schisto+Pk (P = 0.009) and Schisto/PZQ+Pk groups (P = 0.008). Similarly, IgG1 levels in the Pk-only group were higher at third infection compared to baseline (0.0004) and first infection (P = 0.022), as shown in Fig. 5B. At the third infection, IgG (P = 0.01) and IgG1 (P = 0.031) antibodies were higher in the Pk-only group than in the Schisto+Pk group. These data indicate that malaria-specific IgG and IgG1 stimulation is impaired in the presence of a schistosome infection.

Reduced memory T-cell pool in coinfected animals. To determine how *S. mansoni* and *P. knowlesi* coinfection influences acquired immunity, we measured levels of memory T cells (T_{MC}) in baboon peripheral blood mononuclear cells (PBMCs) before and after three *P. knowlesi* infections. PBMCs from 17 animals (Schisto+Pk [n = 5], Schisto/PZQ+Pk [n = 5] and Pk-only [n = 7]) with represented samples at each time point were included in the analysis. Using flow cytometry, T_{MC} were defined as cells expressing the surface markers $CD3^+$ $CD4^+$ $CD45RO^+$ (Fig. 6A) and $CD3^+$ $CD4^-$ $CD45RO^+$ (Fig. 6B), which was used as a measure of $CD8^+$ T cells. We found no significant differences between the three groups

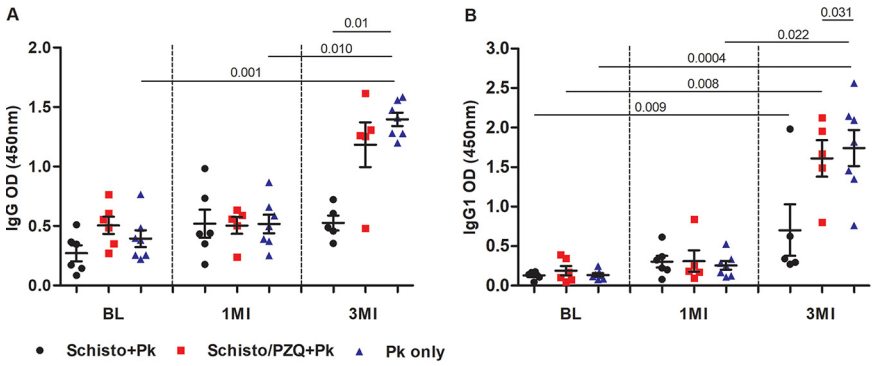

FIG 5 Lower levels of IgG and IgG1 antibody responses in coinfected animals after three *P. knowlesi* infections. The graphs represent IgG (A) and IgG1 (B) antibody responses against malaria before schistosome and malaria infections (BL), after first malaria infection (1MI), and after three malaria infections (3MI). Antibody levels are presented as optical density (OD) measured at a wavelength of 450 nm. Kruskall-Wallis with Dunn's *post hoc* analysis and multiple comparisons adjusted by the Holm method was done for the following data: between-group data at IgG-3MI, IgG1-BL, and IgG1-1MI and within-group data for IgG-Schisto/PZQ+PK, IgG-Pk-only, and IgG1 in all three groups. Between-group data for IgG-BL, IgG-1MI, and IgG1-3MI and within-group IgG-Schisto+Pk data were analyzed using one-way ANOVA with Tukey's multiple comparisons of means.

before or after three *P. knowlesi* infections in either $CD4^+$ T_{MC} or $CD8^+$ T_{MC} levels. However, there were significant reductions in $CD4^+$ T_{MC} levels in the Schisto+Pk ($P = 0.042$) and Schisto/PZQ+Pk (0.045) groups after the third infection compared to those before infection. In contrast, there were no significant differences in expression of $CD8^+$ T_{MC} within or between groups before or after three infections. These data show that chronic exposure to schistosome infection (ongoing or previous) may lead to reduction in the development of T_{MC} to subsequent *P. knowlesi* infection.

Increased CD14 and CD16 expression in animals exposed to *S. mansoni* infection. To further explore innate immune responses during schistosome and malaria coinfection, we determined the expression of $CD14^+$ and $CD16^+$ on PBMCs of 17 animals, as mentioned above. Using flow cytometry, total $CD14^+$ was defined as cells expressing $CD3^-$ $CD14^+$ (Fig. 7A) and nonclassical monocytes (NCM) as $CD3^-$ $CD14^-$ $CD16^+$ (Fig. 7B). We recorded increased levels of $CD14^+$ after the third infection compared to those before infection in the Schisto/PZQ+Pk group ($P = 0.020$). There was also significant increase in NCM levels after the third infection in the Schisto+Pk ($P = 0.047$) and Schisto/PZQ+Pk ($P = 0.029$) groups compared to those before infection. There were no significant differences between the three groups in expression of both $CD14^+$ and

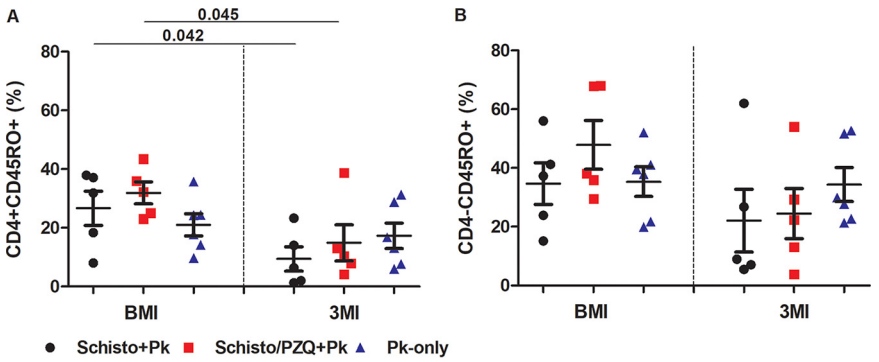

FIG 6 Decreased levels of memory T cells in coinfected animals after three *P. knowlesi* infections. Memory T cells were characterized as the percentages of $CD3^+$ cells expressing $CD4^+$ $CD45RO^+$ (A) and $CD4^-$ $CD45RO^+$ (B). Comparisons were done between the Schisto+Pk (black circles), Schisto/PZQ+Pk (red squares), and Pk-only groups (blue triangles) before malaria infection (BMI) and after three malaria infections (3MI). Between-group data were analyzed by one-way ANOVA with Tukey's multiple comparisons. Within-group data were analyzed by two-sample *t* test.

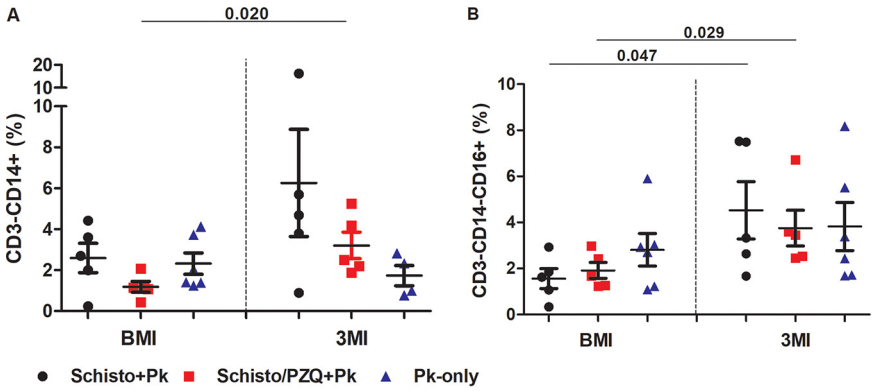

FIG 7 Increased CD14 and CD16 expression in PBMCs of coinfected animals after three *P. knowlesi* infections. $CD3^-$ $CD14^+$ (A) and $CD3^-$ $CD14^-$ $CD16^+$ (B) were expressed as percentage of total lymphocytes. Comparisons were made between the Schisto+Pk (black circles), Schisto/PZQ+Pk (red squares), and Pk-only groups (blue triangles) before malaria infection (BMI) and after three malaria infections (3MI). Differences between the three groups were analyzed using Kruskal-Wallis with Dunn's *post hoc* analysis and multiple comparisons adjusted by the Holm method at 3MI-CD14, 1MI-CD16, and 3MI-CD16. Differences between the three groups at BMI-CD14 was measured by one-way ANOVA with Tukey's multiple comparison. Within-group data were analyzed as follows: $CD14^-$ Schisto+Pk, $CD16^-$ Schisto+Pk, and $CD16^-$ Schisto/PZQ+Pk by Wilcoxon rank sum test and $CD16^-$ Schisto/PZQ+PK, $CD14^-$ Pk-only, and $CD16^-$ Pk-only by two-sample *t* test.

NCM. These data show that exposure to *S. mansoni* with an ongoing or previous infection results in expansion of innate immunity that could be associated with clearance of *P. knowlesi* parasites.

DISCUSSION

In regions where malaria is endemic, individuals develop partial immunity to malaria after repeated or chronic infections, characterized by reduction in severity of clinical disease, parasitemia and inflammation (4). Here, we investigated how chronic *S. mansoni* and *P. knowlesi* coinfection impacts naturally acquired immunity to malaria. We demonstrated development of clinical immunity in both singly infected and coinfected baboons after three malaria episodes, with protection from high fever, high parasitemia, and loss of appetite. Human studies looking at schistosome and malaria coinfections have reported similar findings with protection from febrile malaria (24), high parasitemia, and anemia (25, 26). At phase II of the experiment, the five animals that died had low parasitemia, probably due to parasite sequestration in organs as previously observed (18). Three of these animals had whipworms, and such coinfections with schistosomes has been shown to be detrimental and might have contributed to increased pathogenesis and death (27). These conditions may have led to metabolic disturbances, acute septicemia, and pneumonia, leading to death. Although there was no significant association between death and ongoing or previous exposure of animals to *S. mansoni* infection, it was noted that four of the five animals that had fatal outcome were in the experimental groups infected with schistosomiasis.

IgG1 and IgG3 antibody responses to blood-stage parasites play a pivotal role in protection against malaria (8). These IgG subtypes neutralize parasites directly by inhibiting invasion or growth in erythrocytes or indirectly by antibody-dependent cell-mediated inhibition involving monocyte-derived mediators (28, 29). Although both isotypes are indicated in mediating specific killing action, IgG1, instead of IgG3, has been associated with clinical protection against malaria infection (30). After three malaria episodes, we observed significantly lower levels of circulating malaria-specific IgG and IgG1 in coinfected animals compared to levels in those singly infected, indicating that chronic *S. mansoni* infection influences humoral immune responses to malaria antigens. Human *Schistosoma haematobium* and *Plasmodium falciparum* coinfection studies have reported conflicting results. Similarly to our studies, Courtin et al. reported

low IgG levels in coinfected Senegalese residents compared to levels in those who were schistosome free (31). Others in Benin (26) and Senegal (19) reported high levels in the coinfected group. Such variations may be due to differences in species, antigen assays, age, genetic background, environmental factors, and/or transmission intensity.

Immunological memory in any infection is a key feature of antigen-specific responses, and maintenance of T- and B-cell memory pools ensures quick, effective, and specific response to reinfection. During malaria infection, $CD8^+$ T_{MC} have been largely associated with protection against liver-stage infection, while $CD4^+$ T_{MC} are indicated in blood-stage parasite clearance and support humoral responses (32). During schistosome infection and after treatment, studies have shown suppression of $CD4^+$ T_{MC} compared to the uninfected (33). Here, we observed a significant reduction in $CD4^+$ T_{MC} (cells expressing $CD3^+$ $CD4^+$ $CD45RO^+$) and not $CD8^+$ T_{MC} ($CD3^+$ $CD4^-$ $CD45RO^+$) in schistosome-exposed animals (active and treated groups) after three malaria infections, further demonstrating a modulatory effect of bystander antigens as previously reported (20, 34). Unlike our findings, Lyke et al. demonstrated enhanced $CD4^+$ T_{MC} in antigen-stimulated PBMCs of *S. hematobium*- and *P. falciparum*-coinfected children with a dominant IL-4 expression. (21). Differences in study outcome may be a consequence of different cell types studied, differing study designs, and parasite strains.

Monocytes are key innate immune cells produced early during infection and play a critical role in homeostasis and disease pathogenesis (35). Three subsets of monocytes have been described based on expression of CD14 (lipopolysaccharide receptor) and CD16/FcγRIIIA, namely classical ($CD14^+$ $CD16^-$), intermediate ($CD14^+$ $CD16^+$), and NCM/patrolling ($CD14^{lo/-}$ $CD16^+$) cells (36). Classical monocytes are the largest subset and mediate migration to sites of inflammation, where they can differentiate in tissues to macrophages or dendritic cells. NCM, on the other hand, are involved in immune regulation and tissue repair, while intermediate monocytes are most efficient at phagocytosis and activation of IgG or complement opsonization of *Plasmodium*-infected red blood cells (RBCs) (reviewed previously [11, 37]). Here, we looked at the expression of total $CD14^+$ and NCM, both of which were significantly elevated after three *P. knowlesi* infections in groups exposed to *S. mansoni*, unlike in Pk-only infected animals, which showed no change. NCM, as characterized by high CD16 expression, was recorded in healthy individuals compared to children with acute malaria in a Kenyan study, while this expression was associated with lower severity of *P. falciparum* infection and better survival outcomes in patients in Benin. In contrast with our findings, these studies did not include the asymptomatic or the influence of coinfections, considering that the study populations were from regions where *Schistosoma* is endemic (38, 39). During chronic schistosomiasis, elevation of NCM in monkeys and of $CD14^+$ and monocyte subsets in humans has been recorded (40–42). The expansion of NCM and $CD14^+$ noted in coinfected animals may be associated with exposure to schistosome infection and may play a role in protection from severe disease by inducing an anti-inflammatory immune response and facilitating phagocytosis of dying endothelial, cells thus maintaining vascular homeostasis (35).

In conclusion, our study indicates that acquired immunity to malaria in baboons coinfected with *P. knowlesi* and chronic *S. mansoni* is differentially altered compared to that in animals infected only with malaria. Although all groups of animals exhibited immunity to clinical malaria, coinfection was associated with reduced levels of parasite-specific IgG and IgG1 antibodies and $CD4^+$ T_{MC}. Despite this, speculatively, these levels may be sufficient to mount protective responses. Increased levels of NCM imply a role of the innate immune system. The question of how reductions in protective antibody and $CD4^+$ T_{MC} levels impact long-term acquired immunity to malaria needs additional study. Our study had several limitations, as follows. (i) Four of five animals that died at the second phase of the study were exposed to schistosome infection. Although three of these animals died within 10 days of the first malaria inoculation, illustrating the heterogeneity of the study, we were unable to determine the effect of prolonged chronic

schistosome infection on acquired malaria immunity. (ii) Due to technical limitations, our flow cytometry panels for both T cells and monocytes were not exhaustive, and we were unable to either quantify and compare classical and intermediate monocytes across the groups or further characterize memory T-cell subsets. (iii) Although this study demonstrated differential generation of memory T and innate immune cells, we did not stimulate the PBMCs with specific parasite antigens that would further delineate the role of chronic schistosomiasis in the development of acquired immunity to malaria infection. Further understanding of the differential roles of monocyte subsets and the Th1 and Th2 milieu and how the immune system may impact physiological changes could lead to a better understanding of the complex interactions during malaria and schistosome coinfection.

MATERIALS AND METHODS

Parasites. *S. mansoni* parasites were obtained from chronically infected baboons maintained at the Institute of Primate Research (IPR). Cryopreserved *P. knowlesi* strain H (originally clone Pk1 [A+] and passaged in rhesus monkeys) parasites were used for malaria infection (43).

Animals. Wild-caught male (n = 10) and female (n = 14) olive baboons (*Papio anubis*) weighing between 4.9 and 11.5 kg were obtained from the Mount Kenya region, Kenya. Animals were housed at the IPR in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) in outdoor group cages and isolated once a week for collection of stool samples to monitor schistosome infection. Animals free of schistosome and *P. knowlesi* infections were included in the experiment. Screening was done by detection of (i) anti-*S. mansoni* and anti-*P. knowlesi* antibodies in serum by enzyme-linked immunosorbent assay (ELISA), (ii) the *Plasmodium* 18S rRNA gene through PCR, and (iii) schistosome eggs in stool samples by microscopy. Malaria inoculation was carried out in a biocontainment unit fitted with both single and double cages to allow normal social behavior. Animals were moved to the biocontainment unit 4 weeks prior to malaria infection to allow them to acclimatize to the new environment. All animal housing had access to natural light and dark cycles with unlimited access to clean water. Animals were fed on nutrition-dense monkey cubes (Unga Farm Care, Ltd., Nairobi, Kenya) supplemented with fruits and vegetables.

Infection and treatment protocol. Twenty-four baboons were randomly assigned to three groups (n = 8, Fig. 1); two groups, Schisto+Pk and Schisto/PZQ+Pk, were percutaneously infected with 500 *S. mansoni* cercariae as previously described (44, 45). The Schisto/PZQ+Pk group was treated with three doses of praziquantel (Biltricide, 80 mg/kg; Bayer Schering Pharma) at weeks 14, 15, and 16 (chronic phase), when schistosome adult worms reach maturity and release eggs, leading to tissue pathology. Schistosome infection and treatment were monitored weekly by stool examination for eggs using the Kato-Katz technique (46, 47).

The two groups, plus a new malaria control group (Pk-only), were inoculated with 10^5 *P. knowlesi*-infected erythrocytes through intravenous (i.v.) injection of the saphenous vein in two phases (23). Each phase had 12 animals, 4 from each of the 3 groups. Three *P. knowlesi* infections were done at weeks 23, 32, and 37 in phase I and at weeks 42, 50, and 55 in phase II. After each infection, parasitemia was assessed daily and animals monitored for signs of clinical malaria. Animals were treated with the antimalaria drug artemether/lumefantrine (Coartem dispersible; Novartis Pharmaceuticals Corporation, East Hanover, NJ) upon acquiring parasitemia of 2% and/or presented with any clinical signs of malaria. Artemether/lumefantrine (20 mg/120 mg per tablet) was given at a dosage of 2 tablets (5 to 6.9 kg), 3 tablets (7 to 10.9 kg), or 4 tablets (>11 kg) twice a day for 3 days. During treatment, animals were sedated using ketamine/xylazine, and Coartem was administered orally by gavage (48). Animals were injected with atipamezole (100 μg/kg body weight; Antisedan) to resuscitate and reverse the effects of the anesthesia.

At 14 to 20 one days after the third *P. knowlesi* challenge, baboons in the Pk-only group were treated with Coartem to clear the malaria infection, while animals in the Schisto+Pk and Schisto/PZQ+Pk groups were euthanized and exsanguinated by portal vein perfusion to recover adult schistosome worms. Euthanasia was induced by deep anesthesia (i.v. injection of sodium pentobarbitone [Euthanaze; Bayer HealthCare] at 100 mg/kg). Blood for serum was collected before schistosome infection (week 0), before *P. knowlesi* infection (week s20 and 40 for phases I and II, respectively) and after three *P. knowlesi* infections (weeks 40 and 57). PBMC isolation for immunoprofiling was done before malaria infection and after three malaria infections.

Clinical assessment. *P. knowlesi* infection was monitored daily by clinical assessment and determination parasitemia levels. Blood from a finger-prick was collected from day three and parasitemia determined by microscopy. Parasitemia was calculated as the number of parasitized red blood cells (iRBCs) in 10,000 RBCs $\times$ 100. Parasite density during the infection period was measured by calculating the area under the curve (AUC) up to the time of treatment. Animals not treated were followed to day 12 postinfection. Clinical symptoms assessed included loss of appetite as measured quantitatively by observing the amount of food eaten by the animal (good, >70%; fair, 40 to 70%; poor, <30%), fever as observed by raised fur and a body temperature of $\geq$37°C, and lethargy by determining the demeanor of the animals and recording it as bright and active or lethargic (dull and listless). Membrane pallor of the conjunctiva and oral mucosa was used as a proxy to measure anemia and was graded as pink and moist (normal), slightly pale (mild anemia), and pale (severe anemia). Other symptoms included vomiting,

bloody urine indicative of hematuria, and labored breathing (suggesting tachypnea). Malaria treatment was administered when animals had parasitemia of >2% and/or showed any clinical symptoms.

Antibody assay. Circulating immunoglobulin G (IgG) and IgG1 against *P. knowlesi* antigen was measured by ELISA. Antigen was prepared from whole *P. knowlesi* asexual-stage lysate (Pkh) diluted in 1× phosphate-buffered saline (PBS; Fisher BioReagents). Ninety-six-well ELISA plates (Immulon 4 HBX; USA) were coated with 50 μL/well of 10 μg/mL Pkh antigen in PBS and incubated overnight at 4°C. Plates were blocked with 100 μL/well of 0.3% bovine serum albumin (BSA; Sigma-Aldrich, Dorset, UK) in PBS-0.05% Tween 20 (PBS-T) for 1 h at 37°C. Aliquots of serum samples (50 μL/well) diluted at 1:400 in 0.5% BSA in PBS-T (diluent) were added in duplicates. Positive (reactive serum) and negative (diluent) controls were included, and plates were incubated overnight at 4°C. Plates were washed 6 times with PBS-T and incubated at 37°C for 90 min with 50 μL/well of horseradish peroxidase-conjugated sheep anti-human IgG and IgG1. After further washing, 50 μL/well of the substrate 3,3′,5,5′-tetramethybenzidine (TMB; Kirkegaard and Perry Labs, Gaithersburg, MD) was added, and plates were incubated at room temperature. After 20 min, 50 μL/well of stop solution (0.5 M sulfuric acid; Merck Millipore) was added and absorbance read at 450 nm using a microplate reader (ELx808; Biotex). Mean antibody levels were expressed as optical density (OD) and recorded as net antibody production (mean of duplicate samples minus mean of negative control). Levels greater than 2SE of the mean of baseline was considered a positive response. All antibodies used were purchased from The Binding Site (Birmingham, UK).

Flow cytometry. Frozen peripheral blood mononuclear cells (PBMCs) were thawed at 37°C, washed twice with 1× PBS, resuspended in culture medium, and rested for 2 h at 37°C/5% CO_2. Viability of cells was determined by microscopy after staining with trypan blue. Fluorochrome-conjugated mouse anti-human monoclonal antibodies were used to stain 500,000 PBMCs in 100 μL of fluorescence-activated cell sorting (FACS) buffer. Two panels were designed to characterize (i) memory T cells with fluorescein isothiocyanate (FITC)-CD3, PerCP-CD4, and phycoerythrin (PE)-CD45RO antibodies and monocyte populations with peridinin chlorophyll protein (PerCP)-CD3, FITC-CD14, and allophycocyanin (APC)-CD16 antibodies (Becton and Dickinson [BD] Biosciences, San Jose, CA). Cells were stained for 25 min, washed, and suspended in 500 μL of FACS buffer. Cell were read on a BD FACSCalibur instrument and data analyzed using FlowJo v10.6.0 software (BD, Ashland, OR). The gating strategy is shown in Fig. S1 in the supplemental material.

Statistical analysis. Statistical analysis was performed using R v3.6.1 (Table S1). Morbidity after each malaria infection was presented as percentages and differences across groups analyzed by chi-square test. Variables that were considered included appetite, fever, color of mucous membrane, demeanor, presence or absence of dyspnea, and hemoglobinuria. The effect of prolonged schistosome infection on severe malaria was analyzed using a log-rank test. To determine whether there were differences in parasitemia and antibody responses between the three groups, Kruskall-Wallis with Dunn's *post hoc* analysis and *P* values adjusted for multiple comparisons by the Holm method was used in data that was nonnormal, and one-way analysis of variance (ANOVA) with Tukey's multiple comparisons of means was performed on data with a normal distribution curve. Differences within groups was measured by the Wilcoxon sum rank test for data following a nonnormal distribution and by the two-sample *t* test for data with normal distribution. To measure normality of the data, the Shapiro-Wilk test was used. Variance was measured by Bartlett's test (normally distributed data) or Levene's test (nonnormal distribution), and data with variance were subjected to the Welch two-sample *t* test. Values were considered significantly different when the *P* value was <0.05.

Ethical statement. This study was approved by the IPR Institutional Science and Ethics Committee and Animal Care and Use committee (IPR/IRC), Nairobi, Kenya (study IRC/12/2008). The IPR/IRC is nationally registered by the National Commission for Science, Technology and Innovation, Kenya.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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